AD________________

GRANT NUMBER DAMD17-96-1-6170

TITLE: Use of p53 Mutation Analysis for Staging Breast Cancer

PRINCIPAL INVESTIGATOR: Christine Carter, Ph.D., M.P.H.

CONTRACTING ORGANIZATION: OncorMed, Incorporated
Gaithersburg, Maryland 20877

REPORT DATE: August 1997

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1997	Annual (1 Aug 96 - 31 Jul 97)

4. TITLE AND SUBTITLE
Use of p53 Mutation Analysis for Staging Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6170

6. AUTHOR(S)
Christine Carter, Ph.D., M.P.H.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
OncorMed, Incorporated
Gaithersburg, Maryland 20877

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Mutations in p53 are found in approximately half of all cancers in man. Their presence, location and molecular characteristics reveal important clues about the etiology of the cancer, the specific DNA transformations that occur, and the spread of disease. The presence of p53 mutations in draining lymph nodes suggests the tumor has already spread systematically. Mutations in the p53 gene have shown to be correlated with outcome and may have direct relevance for staging breast cancer. We used direct sequencing of the p53 gene for mutations in the tumor, and when found, to perform oligonucleotide hybridization of the DNA from the margins and matched lymph nodes draining the primary tumor. Our approach involves the examination of archival specimens from a sample of node-negative breast cancer cases from the American Registry of Pathology, Armed Forces Institute of Pathology. Primary tumor DNA and sequence exons 5-9 of the p53 gene (the region accounting for 95% of the known mutations) are amplified. For those patients with a mutation, the surgical margins and matching nodal tissue DNA are amplified and tumor-specific DNA probes are created to detect the presence of mutant p53 cells.

14. SUBJECT TERMS Breast Cancer
Molecular assay, p53 mutations, micrometastasis

15. NUMBER OF PAGES
23

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature Date

8/21/97

TABLE OF CONTENTS

ANNUAL REPORT
Grant #DAMD17-96-1-6170
"USE OF P53 MUTATION ANALYSIS FOR STAGING BREAST CANCER"

SECTION I--INTRODUCTION

In order to cure cancer through the surgical removal of a breast tumor, it is necessary to remove all malignant cells. Until very recently, the only method of testing the effectiveness of the surgery has been to examine tissue samples taken from around the tumor site, or from the patient's lymph nodes, microscopically for visual evidence of remaining malignant cells. Despite negative pathology, tumors recur about 30% of the time, indicating that surgery was not successful. Evidence is accumulating that molecular micrometastasis assays, rather than traditional light microscopic tissue examination, are more sensitive methods of determining the presence of remaining cancer cells in the surgically resected tumor and lymph node tissue. The presence of cancer cells in the lymph nodes of resected tissue is a leading prognostic indicator of later recurrence and contributes to the clinical staging of disease. A molecular staging assay ("TumorCheck™") has been adapted for routine clinical use by Oncormed, Inc. through license agreements with the developers of this technology. We propose to apply this approach, for the first time, to staging breast cancer.

Mutations in p53 are found in approximately half of all cancers in man. Their presence, location and molecular characteristics reveal important clues about the etiology of the cancer, about the specific DNA transformations that occur, and about the spread of disease. The presence of p53 mutations in draining lymph nodes suggests that the tumor has already spread systematically. Mutations in the p53 gene have been shown to be correlated with outcome and therefore may have direct relevance for staging breast cancer. Immunohistochemical methods for p53 protein accumulation have been used as an indirect measure of the presence of p53 mutations in tumor tissue. These methods are limited in that they only examine the primary tumor, have a low sensitivity, utilize a variety of reagents and antibodies, and as a rule are difficult to compare across studies. To improve the precision and sensitivity, we plan to use direct sequencing of the p53 gene for mutations in the tumor, and when found, perform oligonucleotide hybridization of the DNA from the matched lymph nodes draining the primary tumor. These methods are a precise means for detecting micrometastasis and, therefore, determining the success of surgery.

We will examine archival specimens from 100 node-negative breast cancer cases from the American Registry of Pathology, Armed Forces Institute of Pathology. To avoid potential bias, we have controlled for stage (Stage I patients only) and length of follow-up (minimum of 14 years) and we are comparing samples (n=50) that died during the interval with those (n=50) still alive after 14 years of follow-up. We plan to amplify the primary tumor DNA and sequence exons 5-9 of the p53 gene (the region accounting for 95% of the known mutations). For those patients with a mutation, we plan to amplify the surgical margins and use matching nodal tissue DNA and create tumor-specific DNA probes to detect the presence of mutant p53 cells. Initial studies indicate that this assay for p53 mutations has the capacity to detect 1 cancer cell in 10,000 normal cells, compared to light microscopy which is estimated at 1 in 20 cells.

This innovative approach to detecting micrometastases is expected to be a significant improvement over conventional methods to stage breast cancer. Thus, the molecular staging assay is an extremely promising approach and has direct clinical relevance for the following reasons: p53 mutation studies 1) can identify genetic alterations involved in the origin and progression of tumorigenesis, 2) can reveal micrometastasis, where traditional methods have failed, 3) can function as a molecular dosimeter of carcinogen exposure, and 4) can have a significant impact on cancer management, particularly in the Stage I setting where it is important to determine which early-stage patients should receive aggressive adjuvant therapy and which could be safely treated with surgery alone.

Background

1. Clinical Utility of p53 Mutation Analysis

Mutations in the tumor suppressor gene p53 are found in approximately half of all cancers in man. Hence, they are the most common, specific, genetic alteration found in human cancers. About 50% of the 6.5 million cancers diagnosed each year have p53 mutations. These mutations are found in both inherited and sporadic forms of many cancers. Their presence, location, and molecular characteristics can reveal important clues about the etiology of the cancer and about the specific DNA transformations that occur. Moreover, the frequency and type of mutations can provide sensitive information about the degree of exposure to certain known carcinogens, such as dietary aflatoxin B in liver cancer, and cigarette smoke in lung and head and neck cancer. The tumor suppressor gene is located on chromosome 17 and has been regarded as the "guardian of the genome" because of its key role in the body's anti-tumor defense repertoire and its ability to arrest cell growth for DNA repair. When mutated, these advantages are lost and the cell is often thrown into uncontrolled growth which can lead to cancer. Mutations affecting the p53 gene are primarily missense mutations that occur 95% of the time in one of five evolutionary conserved regions (exons 5-9) of the gene (Soussi et al, 1990, Baker and Vogelstein, 1992). More than 51 types of human tumors with p53 mutations have been documented to date. Among the common tumors, 70% of colorectal, 50% of lung and head and neck, and 30-35% of breast cancers contain p53 mutations. Tumors with mutations in p53 are more aggressive, contribute to a shorter disease-free interval, and are resistant to standard treatment regimens (Thor et al., 1992).

Over the past few years, immunohistochemical studies have been performance to indirectly analyze for the presence of p53 mutations. These methods are, however, somewhat problematic and consistency is difficult to demonstrate across studies since a variety of reagents, antibodies and methods have been used. Moreover, differences in specimen integrity further complicate the interpretation of results. Direct sequencing for p53 mutations is now available, and this method has been shown very recently to have dramatic, clinical implications in the management of patients with head and neck cancer. In the past two years, Dr. David Sidransky and a team of researchers at Johns Hopkins University developed a molecular method of tumor staging, utilizing p53 mutation analysis, which we now plan to apply for the first time to breast cancer.

Since the research on head and neck cancer conducted at Johns Hopkins University (Brennan, et al., 1995) is so germaine to this proposal, we herein summarize the results of the study. These investigators examined 72 margins (average of 2.6 margins per patient) amongst 25 patients with no evidence of microscopic carcinoma. In 13 of the 25 patients (52%) the amplified p53 region from at least one surgical margin hybridized to the tumor specific probe, indicating the presence of neoplastic cells containing p53 mutations. Of the 28 lymph nodes available (an average of 5.5 nodes per patient) that were negative by light microscopy, 6 (21%) were found by molecular analysis to contain neoplastic cells with p53 mutations. Moreover, in 5 of the 13 patients (38%) with positive margins by mutation analysis, the cancer recurred locally, as compared with 0 of the 12 patients with negative margins. These results were found to be statistically significant.

The proposed study presents an opportunity to conduct an important validation study of the p53 mutation analysis for staging breast cancer. Since p53 mutation analysis can detect one malignant cell in 10,000 normal cells (1:10,000), and standard light microscopy can only detect 1:20 cells, this technique should significantly improve upon conventional approaches to detecting metastases and provide a means of assessing the success of surgery. The examination of frozen sections of lymph nodes is a critical step in staging cancers, and as many as 30% of tumors with apparently benign lymph nodes are known to have a local recurrence. The application of molecular methods will help stratify patients more precisely. It is important to identify the subset of patients for whom molecular analysis reveals the presence of cancer cells even after pathologic examination showed the surrounding tissue and lymph nodes free of disease. These patients are at risk for local recurrence and are therefore candidates for adjuvant chemotherapy. It is also important to identify the subset of patients who clearly, by both molecular and standard pathologic examination, have no detectable tumor in the lymph nodes. These patients are free of disease due to their curative surgery. Hence, the cancer management implications are enormous when the molecular staging assay is added to the standard treatments options available to patients with early-stage disease.
To summarize, evidence is accumulating to suggest that p53 could potentially be extremely useful in clinical oncology for the following reasons.

p53 mutation studies can be implemented:

- to screen at-risk patients and to facilitate early diagnosis,
- to monitor the spread of disease through molecular staging and to establish prognosis,
- to tailor treatment of individual patients and to target management,
- to assess response to therapy and to associate treatment and outcome to a particular molecular marker, and
- to distinguish subsets of patients as candidates for entry into treatment trials designed to assess optimal strategies for management of early stage disease.

Oncormed, Inc. has adapted the molecular staging assay developed by Dr. David Sidransky for routine clinical use. As a commercial firm, we develop and market medical services utilizing recent genetic discoveries for the early detection and management of cancer. We have direct, relevant experience and expertise in the application of p53 mutation studies. Dr. David Sidransky, a member of Oncormed's Scientific Advisory Board (attached in Section 3.G.), has

participated with us in a cooperative clinical study (detailed in Section 3.G.) on head and neck cancer which facilitated the further development of these technologies into a directly marketable service known as "TumorCheck™" (Oncormed's proprietary name). As a result of this collaboration, Oncormed entered into a sublicense agreement with our parent-affiliate Oncor, Inc. which holds the exclusive world-wide rights to this technology through a license with Johns Hopkins University.

To our knowledge, the proposed study would be the first application of these methods to staging breast cancer, and would provide valuable information to validate the techniques for staging in a critically important and prevalent cancer. We anticipate that this technology, once validated and implemented in the marketplace, will surpass conventional, histologic examination which is inaccurate 30% of the time. This molecular staging assay will address why node negative or tissue margin negative patients are relapsing at a rate of (coincidentally) 30%. We plan to validate the utility of these methods beyond head and neck cancer, to collect empirical evidence that these technologies are an enhancement to conventional means of cancer surveillance, and to assess their clinical utility on a case-by-case basis for the management of early-stage breast cancer patients.

2. p53 Mutation Studies for Staging Breast Cancer

The frequency or spectrum of p53 mutations in breast cancer patients varies by a number of factors, including certain epidemiologic factors, carcinogen exposures, or inherited features. p53 mutations have been reported in as few as 22% (Thor et al., 1992) but as many as 40% (Coles et al. 1992; Blaszyk et al., 1994) of primary breast cancer tumors; with the average about 30-33% (Tsuda et al., 1993). In most studies examining the utility of p53 as a prognostic indicator, immunohistochemical (IHC) analyses has been performed. IHC is an indirect method of measuring p53 protein accumulation using a variety of techniques, specimens, reagents, and antibodies and, thus, as a rule, this method has been difficult to compare across studies. The direct method of sequencing the p53 gene for mutations is far more accurate and has a sensitivity of approximately 90-95% and specificity >99%. The majority (95%) of the mutations found in the p53 gene are in exons 5-9 (a "hotspot" region), which are considered essential domains for the function of p53. Studies on the clinical utility of assays for p53 mutations indicate that the presence of a mutation(s) is correlated with significantly reduced time to recurrence, disease-free survival, and overall survival (Thor et al., 1992; Blaszyk, et al., 1994, Greenblatt et al., 1994). Particularly compelling, we find, are studies that suggest the potential for p53 mutation analyses in reaching treatment decisions for lymph node-negative breast tumors (Isola et al., 1992; Silvestrini et al., 1993; Thor and Yandell, 1993).

The management of early-stage breast cancer presents difficult treatment options. Only about one half of patients with primary operable breast cancer will either sustain long-term remission or be cured by their surgery. The strongest predictor of recurrence is the presence of tumor in the axillary lymph nodes, yet, still, 30% of women whose nodes are free of disease will recur (Elledge et al., 1992, Callahan, 1992). Tumor size and lymph node status remain powerful predictors of recurrence and death; however, the relationship between size and outcome is not strictly linear. A certain percentage of very small tumors (<1cm) have an appreciable incidence

(22%) of metastasis, which indicates that size and metastatic potential are not perfectly correlated (Carter, et al., 1989). It is plausible that p53 mutations are present in the margins of these small, aggressive tumors. The approach we propose, to detect p53 mutations in the tumor and in matched lymph node materials, should provide important staging information regarding micrometastasis. In doing so, it will provide a means to distinguish patients who would benefit from receiving adjuvant therapy, despite their apparent node negative status and small tumor size, from those that could be spared unnecessary chemotherapy.

The search for new predictive and prognostic indicators for axillary node-negative breast cancer patients is ongoing (McGuire, et al., 1990, Gasparini, et al., 1993, Davidson, 1990, Elledge, et al., 1992). The markers under study include hormone receptors, tumor cell proliferation rate, DNA ploidy, EGFR, and c-erbB-2 gene amplification or expression. Recently, the requirements of an "ideal" profile for a prognostic indicator for node-negative breast cancer were published (Gasparini et al. 1993). None of the above-referenced markers meet these criteria. These investigators suggest that the markers should: 1) be the expression of a biological phenomenon related to tumorigenesis, 2) be detectable by a feasible, reproducible and sensitive method, 3) be independently predictive, 4) be able to distinguish subgroups of at-risk patients, 5) be easily interpretable by the clinician, and 6) be an improvement over existing conventions to select a subgroup for therapy.

Mutations in p53 meet, potentially, all of these criteria. p53 mutations are associated with genomic instability, increased proliferative rates and aneuploidy, and increased susceptibility to cancer (Greenblatt et al., 1994). With direct sequencing of the p53 gene for mutations, the reliability, sensitivity and predictability of p53 as a marker for recurrence far exceeds immunohistochemistry (MacGeoch et al. 1993; Jacquemeier et al., 1994; Sato et al., 1995) and conventional pathologic evaluation (Brennan et al., 1995). By applying p53 mutation analysis in the Stage I setting, it will be possible to distinguish which patients have the potential for metastasis and therefore can be targeted for adjuvant chemotherapy. This method can detect 1:10,000 cells compared to microscopy (1:20 cells). Investigation of the p53 tumor supressor gene will lead to a better understanding of the molecular processes of carcinogenesis and steer the clinician to management strategies which are streamlined, cost-effective and appropriately tailored to the specific needs of a given cancer patient.

3. Additional Application of a Competing Technology

Since it is available from our collaborators and inexpensive, we also propose to perform immunohistochemical analysis of the draining lymph nodes for evidence of cytokeratin expression. Nasser et al. (1993) have demonstrated that demonstration of cytokeratin expression in histologically negative lymph nodes is predictive of recurrence and death in breast cancer patients. We will compare the efficacy of the TumorCheck™ assay with that of this immunohistochemical assay in predicting adverse outcome.

4. The American Registry of Pathology

The specimens for this research will be obtained through a subcontract with our collaborators Drs. Timothy O'Leary and Michael Peterson from the Armed Forces Institute of Pathology (AFIP). The AFIP founded in 1862 and located at the Walter Reed Army Medical Center in Washington, DC, is one of the world's leading institutes for the study of pathology. The Institute utilizes over 700 United States Army, Navy, Air Force and Public Health Service personnel, including members of the Civil Service and Department of Veterans Affairs. The AFIP provides services to all military, Veterans Affairs, Centers for Disease Control, National Aeronautics and Space Administration, and other government agencies, along with the worldwide civilian medical community. The organization has as its major professional activities consultation, education and research in pathology. The American Registry of Pathology serves as the repository of the institute, and stores over 2.5 million cases since 1917 (paper records, 50 million slides, 30 million paraffin blocks and 12 million tissue specimens). Approximately 50,000 cases are accessioned and coded each year. Approximately 1000 breast cancer cases, diagnosed between 1970-1980, have been previously studied and are well characterized with respect to contributing source [(e.g. the Department of Defense, Veterans Administration, civilian (fed), and civilian (non-fed)]; race (white, black, other), vital status and other epidemiologic factors. The cases have been followed for up to 14 years. For this study, 100 Stage I breast cancer cases will be selected (50 who have died during the follow-up period, and 50 who were still alive at the end of the follow-up period).

Hypothesis/Purpose

The primary purpose of this study is to assess the effectiveness of p53 mutation analysis to detect the spread of tumor cells in the lymph nodes of excised tissue from Stage I breast cancer patients. In addition we plan to examine the lymph nodes for the presence of cytokeratin. The specific hypotheses include:

1. The p53 molecular staging assay will detect the presence of p53 mutations, indicative of micrometastasis, in the draining lymph nodes of archival breast cancer specimens from Stage I breast cancer patients.

2. The p53 molecular staging assay is more sensitive that conventional histological examination for the detection of tumor cells in the draining lymph nodes of excised tissue from Stage I breast cancer patients.

3. Immunohistochemical analysis for cytokeratin expression will indicate the presence of micrometastasis in the draining lymph nodes of archival breast cancer specimens from surgically resected, conventionally treated, Stage I breast cancer patients.

4. The presence of micrometastases, as demonstrated by molecular staging and/or cytokeratin staining, indicates a worse prognosis for patients who are node-negative by conventional histologic examination.

5. Molecular and/or cytokeratin staining for tumor metastasis are effective techniques for staging breast cancer.

Original Statement Of Work

Task 1. At AFIP, identify a sample of 100 Stage I breast cancer cases for molecular and biological determination of micrometastasis. Month 1.

a. Select from the American Registry of Pathology database, only node-negative cases with tumor size <2 cm. From these, select 50 cases that were still alive at the end of the 14 year follow-up period and 50 cases that died during the follow-up period.

b. Conduct all retrieval and re-examination activities to ensure tissue- and record-availability on the 100 cases.

Task 2. At AFIP, retrieve archived materials and re-cut a sample (≥5 microns) of tissue from the paraffin-embedded blocks from the primary tumor from each of the 100 cases, label, store in small vials, and batch for transport to Oncormed for analysis. Months 2-3.

Task 3. At Oncormed, conduct molecular studies using TumorCheck™ for the detection of micrometastasis in the primary tumor. Months 4-12.

a. Amplify the DNA by standard polymerase chain reaction (PCR) methods.

b. Perform direct sequencing of DNA in exons 5-9 of the p53 gene for mutations.

Task 4. Conduct further analyses on the lymph node material from approximately 30-35 cases where a mutation in the p53 gene has been identified in the primary tumors. Months 11-16.

a. At AFIP, retrieve all matched specimens available from the archived lymph node material for those cases where a mutation has been identified (could be 1-20 nodes), and re-cut a sample (≥5 microns) from each of the available paraffin-embedded blocks. Label, store and batch for transport to Oncormed for additional analyses.

b. At Oncormed, perform oligo-specific hybridization studies on the amplified DNA from each of the lymph node specimens for evidence of the p53 mutation(s).

c. At AFIP, perform immunohistochemical analyses for cytokeratin.

Task 5. At Oncormed, analyze, present and publish data (or produce all written reports as required). Months 17-24.

a. Perform all statistical analyses to assess the independent contribution of the molecular and biologic markers to the overall survival of breast cancer in this cohort.

b. Produce all written documentation for reports and/or publications as required or desired by collaborators.

Original Technical Objectives

a) Specific aim #1: Identify, from the American Registry of Pathology database, a sample of 100 Stage I (node-negative, tumor size <2 cm.) breast cancer cases who were dead (n=50) or alive (n=50) at the end of fourteen years of follow-up.

b) Specific aim #2: Retrieve and reexamine material (excised paraffin-embedded tumor tissue) from these 100 cases, and re-cut a sample of tissue for subsequent laboratory analysis.

c) Specific aim #3: Amplify the tumor DNA using the polymerase chain reaction (PCR) and conduct DNA sequence analysis of exons 5-9 of the p53 gene for evidence of p53 mutations.

d) Specific aim #4: Retrieve, reexamine and re-cut matched sample(s) of lymph node material for those cases where a mutation was detected, make plasmid libraries of each lymph node DNA and hybridize with a probe specific to each tumor. Conduct immunohistochemical analysis for cytokeratin.

e) Specific aim #5: Perform the appropriate statistical analyses.

SECTION II--BODY

Methods

Genomic DNA will be isolated from the primary breast tumor and subjected to PCR to amplify the p53 gene hotspot (exons 5-9), where 95% of the mutations are found. The PCR product will then be sequenced to identify any tumor-specific mutations which may be present. When a p53 mutation is identified, an oligonucleotide probe specific for this mutation is synthesized. The p53 gene is then amplified from the tissue specimens removed during surgery. These PCR products are then cloned into bacterial cells via a plasmid vector, and plated on agar plates. For each surgical specimen, greater than 10,000 bacterial clones are generated. These "mini-libraries" are then fixed onto nylon membranes. The oligonucleotide probe specific for the mutation identified in the primary breast tumor is then radioactively tagged and hybridized to these membranes. Any filter demonstrating hybridization with the oligonucleotide probe indicates the presence of tumor cells in the surgical specimen represented by that filter.

Immunohistochemical assays for cytokeratin will be performed using standard methods (Frisman et al., 1993).

Statistical analyses will be conducted to assess the independent effects of p53 mutations, presence of cytokeratin in the lymph nodes and other clinicohistologic factors on overall survival from breast cancer. The sample size proposed for this investigation is capable of identifying, with 90% certainty, a three-fold increase in risk of death associated with a positive TumorCheck™ assay result, as compared with the risk associated with a negative result.

Description of Work Accomplished In Year 1

AFIP has retrieved, re-cut and transported to Oncormed 78 of the 100 samples selected for this study.

Sample Preparation. The Study Technologist, Geoff Jackson, prepped 41 samples to date (410 slides @ 10 slides per patient) which were reviewed by our pathologist, Dr. Doug Dolginow.

The following results were achieved at the pathology level (n=41):

Tumor sufficient for Oncormed analysis:	25	(61%)
Insufficient tumor for Oncormed analysis:	16	(39%)

DNA Extraction and Amplification. Using Oncormed's paraffin extraction protocol and PCR amplification methods, twelve (12) samples containing confirmed tumor specimens were analyzed and none (0) of the prepared DNAs amplified with the p53 primers used conventionally at Oncormed, except for 1 of the 5 exons from one (1) sample (see Army Sample Data File, Spec. T960302, attached).

A meeting of the principals from Oncormed (4 individuals) and AFIP (1 individual) involved in this study was convened on February 13, 1997, to discuss the character of the samples and the failure to extract and amplify DNA from the samples with confirmed tumor.

Several decisions were made:

(1) Samples that contained little or no tumor would be returned to our AFIP collaborator, Dr. Timothy O'Leary, who agreed to re-cut sections from the available block and provide Oncormed with suitable replacement specimens containing tumor.

(2) Oncormed would test the procedure for DNA extraction from paraffin used by Dr. O'Leary's lab in an attempt to get the samples to work. Dr. O'Leary provided Oncormed with his published protocol (Reid et al., Diagnostic Molecular Pathology 5:65-73, 1996).

Test Runs using O'Leary protocol. Geoff Jackson used this protocol on 4 samples, and amplification was still unsuccessful (Data File: T960213, T960296, T960302, T960313).

Oncormed then arranged with Dr. O'Leary to have one of the AFIP technicians involved in this research in his lab spend a day at Oncormed with our technologist. During this visit, Ms. Maryam Zavar, who spent several months developing the protocol for extraction of DNA from paraffin for Dr. O'Leary's group, pointed out numerous differences between the published protocol and the one that she had been further developing. All agreed that further runs were required to determine whether or not the Zavar method would be successful.

Test Runs using the Zavar protocol. Geoff Jackson tried the Zavar protocol on 6 of the study samples (Data File: T960247, T960291, T960298, T960299, T960300) and on an additional Oncormed paraffin sample (Data File: C970265) that was recently obtained. The protocol resulted in amplification on the Oncormed clinical sample, but failed on all 6 of the study samples. This indicated that the procedures were being followed accurately, and worked on the fresh specimens and failed on the older samples either because of insufficient DNA or because the DNA was degraded to the extent that samples could only be amplified with primers giving very short PCR products, such as the ones used in the Zavar protocol (and not in Oncormed's lab).

Summary of Unanticipated Problems

It was believed at the beginning of this study that the Army samples would be compatible with Oncormed's protocols and technology. However, three major issues have impacted the progress of this study.

1. The amount of tumor present is less than that usually used by Oncormed (in almost 40% of the prepped samples).

2. Oncormed's protocol and technology have been proven in our clinical facility for a number of years. However, in an attempt to amplify these samples, Oncormed has had to expend time

and resources (including trying two other DNA extraction protocols) beyond what was anticipated for this project.

3. Possible reasons for failure to amplify DNA in these samples are as follows. It seems likely that the failure results from the fact that the amplification products produced by the Oncormed primer sets are too long for specimens this old. Primers producing short p53 amplification products have previously been used at AFIP on breast cancer specimens from this era, and many of these samples have been used in another AFIP study to detect loss of heterozygosity. Redesigning the primers at Oncormed is beyond the scope of work and budget that has been approved for this project. The primers would have to be modified to include the 5' and 3' restriction enzyme sites to facilitate the cloning step of the assay. In addition, in order to generate enough sequencing template, a reamplification of the PCR products will likely be necessary. Reamplification of an amplified product is a concern in any laboratory due to the increased risk of PCR contamination. This is an unacceptable risk for our CLIA-approved facility.

Proposed Solutions to Identified Problems

We believe that the most significant unanticipated problem for this study is that the degree of DNA degradation present in the samples that were provided to Oncormed for analysis precludes effective application of the originally-proposed Oncormed assay.

Oncormed has performed the p53 assay with success for over 2 years, on samples that are less than five (5) years old. The data to support this assertion is included in Table 1 (attached). We propose, that for this study, sample selection be modified to include only samples that are 5 years old or less.

A pilot study of 5-10 samples was performed to test the compatibility of these more recent Army samples with the current Oncormed procedures (the fact that the majority of the samples prepped were between 16 and 26 years old is most likely the reason we are not able to extract adequate amounts of DNA and PCR amplify using the originally-proposed primer sets). We felt that the study's major objective, to apply the previously-published molecular staging assay to breast cancer, for the first time, and to establish the clinical relevance of this assay, was not disturbed by this modification. Comparing the results in a newer cohort of 100 cases with up to 5 years of follow-up, up to one-third of whom have died of their disease during this period was envisioned. Our AFIP collaborator identified this new cohort of individuals, however, a test run of 5 samples from the newer cohort indicated that the DNA was of a poor quality and did not amplify.

Contact With DOD Grants Office

A conference call between Mr. Brian Martin, Contracting Officer, Dr. Patricia Modrow, Technical Officer, Dr. Christine Carter, Principal Investigator, and Ms. Jennifer Breen, Grants Administrator was held on June 13, 1997. The call began with Dr. Carter reviewing the different issues and problems that had been occurring with the samples the AFIP has sent. Dr. Carter went on to explain that Oncormed's protocols, which are designed for commercial

application and are performing as standard in our clinical facility, were repeatedly used on the AFIP samples with no success.

Dr. Carter related that considerable time, effort and resources had been expended on essentially research and development functions with no advancement in the goals of the study. She then made two requests of the agency officials: (1) reimbursement for the costs to date incurred both by Oncormed and AFIP, and (2) permission for Oncormed to identify another source for obtaining new samples in place of AFIP.

Request for Reimbursement

Dr. Modrow informed Dr. Carter that we would be unable to use additional funds (outside of the award amount) to reimburse us for the research and development costs incurred thus far; however, we could use the funds that have already been awarded for this contract. She also instructed that all analyse conducted under this grant to fulfill the scope of work would need to fall under this budgeted amount.

Request to Obtain a New Collaborator

Mr. Martin and Dr. Modrow both agreed that Oncormed could begin looking for another collaborator to obtain new samples in order to continue this important research project.

Guidelines From DOD Technical Officer

A new source of samples has been identified as of August, 1997. Dr. Bruce Ponder, Ph.D., FRCP, is the Director of the Cancer Research Campaign Human Cancer Genetics Group and Professor of Oncology at the University of Cambridge, UK. Dr. Ponder and his research group has expressed an interest in providing Oncormed with the samples we need to continue this project. In August, Dr. Ponder and his team identified samples between 1980-1983: 117 stage I breast cancers, of which 61 are still alive. We are currently waiting to receive test samples which will be evaluated for quality of DNA and to ensure we don't have the same problems we encountered with the AFIP samples. We anticipate that the scope of work and study objectives can be met with the materials provided by Dr. Ponder. An accelerated schedule will be used to analyze the samples to ensure that grant deadlines are maintained.

Conclusions

Dr. Modrow requested (June 13, 1997) that Oncormed prepare and submit the following materials to document these changes:

* Revised Statement of Work (attached)
* Revised budget (to be submitted to CO and TO)
* Outlined strategy for obtaining new samples (attached)

SECTION III--REFERENCES

Soussi T et al., Structural aspects of the p53 Protein in Relation to Gene Evolution. Oncogene, 5:945-52, 1990.

Baker and Vogelstein, The p53 Gene in Human Cancer. Nuclear Processes and Oncogenes, 14:105, 1992.

Thor AD et al., Accumulation of p53 Tumor Suppressor Gene Protein: An Independent Marker of Prognosis in Breast Cancers. Journal of the National Cancer Institute, 84:845-55, 1992.

Brennan et al., Molecular Assessment of Histopathological Staging in Squamous-Cell Carcinoma of the Head and Neck. New England Journal of Medicine, 332:429-35, 1995.

Coles et al., p53 Mutations in Breast Cancer. Cancer Research, 52:5291-98, 1992.

Blaszyk et al., p53 Gene Mutations in Breast Cancers in Midwestern U.S. Women: Null as Well as Missense-Type Mutations are Associated with Poor Prognosis. ASHG, 1994.

Tsuda H et al, p53 Mutations and c-erbB-e Amplification in Intraductal and Invasive Breast Carcinomas of High Histologic Grade. Jpn J Cancer Res, 84:394-401, 1993.

Greenblatt MS et al., Mutations in the p53 Tumor Suppressor Gene: Clues to Cancer Etiology and Molecular Pathogenesis. Cancer Res. Perspective, 1-116, 1994.

Isola J. et al., Association of Overexpression of Tumor Suppressor Protein p53 with Rapid Cell Proliferation and Poor Prognosis in Node-Negative Breast Cancer Patients. Journal of the National Cancer Institute, 84:1109-14, 1992.

Silvestrini R et al., p53 as an Independent Prognostic Marker in Lymph Node-Negative Breast Cancer Patients. Journal of the National Cancer Institute, 85:965-70, 1993.

Thor AD, Yandell DW, Prognostic Significance of p53 Overexpression in Node-Negative Breast Carcinoma: Preliminary Studies Support Cautious Optimism. Journal of the National Cancer Institute, 85:176-77, 1993.

Ellege R et al., Prognostic Factors in Breast Cancer. Seminars in Oncology, 19(3):244-253, 1992.

Callahan R, p53 Mutations, Another Breast Cancer Prognostic Factor. Journal of the National Cancer Institute, 84(11):826-27, 1992.

Carter CL, Allen C and Henson D. Relation of tumor size, lymph node status and survival in 24,740 breast cancer cases. Cancer 63:181-187, 1989.

McGuire W et al., How to Use Prognostic Factors in Axillary Node-Negative Breast Cancer Patients. Journal of the National Cancer Institute, 82(12):1006-15, 1990.

Gasparini G et al., Evaluating the Potential Usefulness of New Prognostic and Predictive Indicators in Node-Negative Breast Cancer Patients. Journal of the National Cancer Institute. 85(15): 1206-19, 1993.

Davidson N, Biology and Prognostic Factors of Breast Cancer. Current Opinion in Oncology, 2:1025-30, 1990.

MacGeoch C et al., p53 Protein Detected by Immunohistochemical Staining is Not Always Mutant. Dis Markers, 11(5-6):239-50, 1993.

Jacquemier J et al., p53 Immunohistochemical Analysis in Breast Cancer with Four Monoclonal Antibodies: Comparison of Staining and PCR SSCP. Br J Cancer, 69(5):846-52, 1994.

Sato T et al., Detection of p53 Gene Mutations in Aspiration Biopsy Specimens from Suspected Breast Cancers by Polymerase Chain Reaction-Single Strand Conformation Polymorphism Analysis. Jpn J Cancer Res, 86(2):140-45, 1995.

Nasser IA et. al., Occult Axillary Lymph Node Metastases in Node-Negative Breast Carcinoma. Human Pathology, 24(9):

Frisman D et. al., Immunocytochemistry in the Differential Diagnosis of Effusions: Use of Logistic Regression to Select a Panel of Antibodies to Distinguish Adenocarcinomas from Mesothelial Proliferations. Modern Pathology, 6(2): 179-84.

SECTION IV--APPENDICES

P53 GENE SEQUENCING PERFORMED ON PARAFFIN-EMBEDDED SAMPLES AT ONCORMED

IDENTIFICATION NUMBER	SAMPLE TYPE	P53 GENE SEQUENCING RESULTS
1683	Paraffin	WT
1866	Paraffin	Codon 248 CGG->CAG
1870	Paraffin	Codon 173 GTG->ATG
1871	Paraffin	Codon 175 CGC->CAC
0007	Paraffin	WT
2	Paraffin	Codon 230 690insTGTAC Stop @ codon 248
3	Paraffin	WT
10	Paraffin	WT (Ex. 6,7 only)
11	Paraffin	Failed
12	Paraffin	WT (Ex. 6,7,8,9 only)
15	Paraffin	WT
16	Paraffin	WT
17	Paraffin	WT
21	Paraffin	WT
25	Paraffin	Failed
26	Paraffin	Codon 266 GGA->GTA
27	Paraffin	Codon 317 CAG->TAG
28	Paraffin	Codon 306 CGA->TGA
29	Paraffin	Codon 220 TAT->TGT
37	Paraffin	WT
41	Paraffin	WT
42	Paraffin	WT
47	Paraffin	WT
48	Paraffin	WT
50	Paraffin	WT
51	Paraffin	WT
57	Paraffin	WT
58	Paraffin	WT
61	Paraffin	Codon 205 TAT->AAT
76	Paraffin	WT
77	Paraffin	WT
80	Paraffin	Codon 248 CGG->CAG
82	Paraffin	WT
84	Paraffin	WT
85	Paraffin	WT
86	Paraffin	Codon 266 GGA->GTA

ARMY SAMPLE DATA FILE

OMI #	Paraffin Sections Prepared	Pathology Read (Dr. Dolginow)	DNA Prep Method Used	PCR amplification pass/fail
T960210	YES	Tumor present	OncorMed	fail
T960211	YES	No tumor present	OncorMed	fail
T960212	YES	Tumor present	OncorMed	fail
T960213	YES	Very little tumor	OncorMed + O'Leary	fail/fail
T960247	YES	Tumor present	M. Zavar	fail
T960248	YES	Tumor present		
T960249	YES	Very little tumor		
T960250	YES	Tumor present		
T960251				
T960252				
T960253	YES	Very little tumor		
T960254				
T960255	YES	Tumor present		
T960256				
T960257				
T960258	YES	Very little tumor		
T960259				
T960260				
T960261				
T960262				
T960263				
T960264				
T960265				
T960266	YES	Tumor present		
T960267				
T960268				
T960269				
T960270				
T960271				
T960272				
T960273				
T960274				
T960275				
T960276				
T960277				
T960278				
T960279				
T960280				
T960281				
T960282				
T960283				
T960284				
T960285				

ARMY SAMPLE DATA FILE

OMI #	Paraffin Sections Prepared	Pathology Read (Dr. Dolginow)	DNA Prep Method Used	PCR amplification pass/fail
T960286				
T960287	YES	Very little tumor		
T960288	YES	Very little tumor		
T960289	YES	Tumor present		
T960290	YES	Very little tumor		
T960291	YES	Tumor present	M. Zavar	fail
T960292	YES	Tumor present		
T960293				
T960294				
T960295	YES	Tumor present		
T960296	YES	Very little tumor	OncorMed + O'Leary	fail/fail
T960297	YES	Tumor present		
T960298	YES	Tumor present	M. Zavar	fail
T960299	YES	Tumor present	M. Zavar	fail
T960300	YES	Tumor present	M. Zavar	fail
T960301	YES	Very little tumor		
T960302	YES	Tumor present	OncorMed/O'Leary	pass (ex7 only)/fail
T960303	YES	Tumor present	OncorMed	fail
T960304	YES	Tumor present	OncorMed	fail
T960305	YES	Tumor present	OncorMed	fail
T960306	YES	No tumor present		
T960307	YES	Tumor present		
T960308	YES	No tumor present	M. Zavar	fail
T960309				
T960310				
T960311	YES	Tumor present	OncorMed	fail
T960312	YES	No tumor present		
T960313	YES	Tumor present	OncorMed/O'Leary	fail/fail
T960314	YES	Tumor present		
T960315	YES	Very little tumor		
T960316	YES	Tumor present		
T960317	YES	Very little tumor		
T960318	YES	No tumor present		
T960319	YES	Tumor present	OncorMed	fail
T960320				
C960265*	YES	Tumor present	OncorMed/M. Zavar	pass/pass
C970086*	YES	Tumor present	OncorMed/O'Leary	pass/fail
Total=78 Army samples	Total sections prepped = 41 @ 10 slides each	Tumor Present = 25 Very Little Tumor = 11 No Tumor = 5	OncorMed = 12 O'Leary = 4 M. Zavar = 6	

"USE OF P53 MUTATION ANALYSIS FOR STAGING BREAST CANCER" REVISED STATEMENT OF WORK

<u>Task 1.</u> At University of Cambridge, identify a sample of 75 Stage I breast cancer tumors for molecular determination of micrometastasis. Year 2, Month 1 (beginning September 1, 1997).

a. Select from the 1980-1983 Addenbrooke database, only node-negative cases with tumor size <2 cm. From these, select approximately half of the cases that were still alive at the end of the 14-17 year follow-up period and half that died during the follow-up period.

b. Conduct all retrieval and re-examination activities to ensure tissue- and record-availability on the eligible cases.

<u>Task 2.</u> At University of Cambridge, retrieve tumors (paraffin-embedded blocks), label and ship to Oncormed for analysis. Year 2, Month 1.

<u>Task 3.</u> At Oncormed, conduct molecular studies using TumorCheck™ for the detection of micrometastasis in the primary tumor. Year 2, Months 4-6.

a. Amplify the DNA by standard polymerase chain reaction (PCR) methods.

b. Perform direct sequencing of DNA in exons 5-9 of the p53 gene for mutations.

<u>Task 4.</u> Conduct further analyses on the lymph node material from an estimated 1/3 of the cases where a mutation in the p53 gene has been identified in the primary tumors (from task three above). Year 2, Months 5-10. On p53 positive cases:

a. At University of Cambridge, retrieve all matched specimens (paraffin-embedded blocks) available from the archived lymph node material for those cases where a mutation has been identified (could be 1-20 nodes). Label, store and batch for transport to Oncormed for additional analyses.

b. At Oncormed, perform oligo-specific hybridization studies on the amplified DNA from each of the matched lymph node specimens for evidence of the p53 mutation(s).

<u>Task 5.</u> At Oncormed, analyze, present and publish data (or produce all written reports as required). Year 2, Months 10-12.

a. Produce all written documentation for reports and/or publications as required or desired by collaborators.

A timeline to depict these activities is provided in Addenda 3.B.

"USE OF P53 MUTATION ANALYSIS FOR STAGING BREAST CANCER" REVISED TECHNICAL OBJECTIVES

a) Specific aim #1: Identify, label and ship from the University of Cambridge Addenbrooke database, a sample of 75 Stage I (node-negative, tumor size <2 cm.) breast cancer cases who were dead (approximately 35-40) or alive (approximately 35-40) at the end of fourteen-seventeen years of follow-up.

b) Specific aim #2: Amplify the tumor DNA using the polymerase chain reaction (PCR) and conduct DNA sequence analysis of exons 2-11 of the p53 gene for evidence of p53 mutations.

c) Specific aim #3: Analyze matched sample(s) of lymph node material for those cases where a mutation was detected, make plasmid libraries of each lymph node DNA and hybridize with a probe specific to each tumor.

d) Specific aim #4: Perform Analysis and submit required reports.